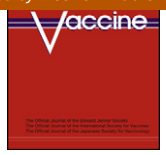
Vaccine 30 (2012) 6678–6685

Vaccine

journal homepage: www.elsevier.com/locate/vaccine



Protective immunity against tick infestation in cattle vaccinated with recombinant trypsin inhibitor of *Rhipicephalus microplus*

Renato Andreotti[a], Rodrigo Casquero Cunha[a], Mariana Aparecida Soares[a], Felix D. Guerrero[b], Fábio P. Leivas Leite[c], Adalberto A. Pérez de León[b,*]

[a] *EMBRAPA Beef Cattle, Avenida Rádio Maia, 830 – Vila Popular – Caixa Postal 154, CEP 79106-550 – Campo Grande, MS, Brazil*
[b] *USDA-ARS Knipling-Bushland U.S. Livestock Insects Research Laboratory, 2700 Fredericksburg Road, Kerrville, TX 78028, USA*
[c] *Universidade Federal de Pelotas (UFPel), Institute of Biology, Department of Microbiology and Parasitology, Rua Gomes Carneiro, 1 – Centro – Caixa Postal 354, CEP 96010-610 – Pelotas, RS, Brazil*

ARTICLE INFO

Article history:
Received 15 April 2012
Received in revised form 1 August 2012
Accepted 28 August 2012
Available online 7 September 2012

Keywords:
R. microplus
Cattle
Tick
Vaccination
Trypsin inhibitor
Recombinant protein

ABSTRACT

The cattle tick, *Rhipicephalus microplus*, is regarded as the most economically important ectoparasite of livestock globally. Control is achieved primarily through the use of acaricides. This approach is hampered by the development of resistance to commercial acaricides among cattle tick populations. Vaccination against *R. microplus* infestation is another technology that can be integrated for effective cattle tick control. Proteins belonging to the Kunitz-BPTI family are abundant in cattle tick salivary glands, midgut, and ovaries. These organs are attractive targets for the development of a novel cattle tick vaccine. Efficacy assessment against cattle tick infestation in bovines using a vaccine containing the recombinant form of a member of the Kunitz family from *R. microplus* produced in a yeast expression system is reported for the first time here. The yeast *Pichia pastoris* was bioengineered to produce the recombinant version of a trypsin inhibitor that is expressed in cattle tick larvae (rRmLTI). Immunization with rRmLTI afforded 32% efficacy against *R. microplus*. The estimated molecular weight of rRmLTI was 46 kDa. Structural homology to the native form of the larval trypsin inhibitor was documented by recognition of rRmLTI in Western-blots using polyclonal antibodies from mice immunized with cattle tick larval extract or rRmLTI. Bioinformatics analysis of the partial nucleotide and deduced amino acid sequences indicated that the rRmLTI closely resembles BmTI-6, which is a three-headed Kunitz protein present in cattle tick ovary and fat tissue.

1. Introduction

Ectoparasitism of cattle by the southern cattle tick, *Rhipicephalus microplus*, inflicts severe economic losses to the livestock industry. Cattle productivity is undermined by the direct effects of ectoparasitism and indirectly by the role *R. microplus* plays as vector of the infectious agents causing bovine babesiosis and anaplasmosis [1,2]. The control of *R. microplus* is achieved mainly through the use of chemical acaricides [3]. However, chemical acaricides have not been utilized judiciously. This has led to the development of acaricide resistance among populations of *R. microplus* [4,5].

Vaccinating cattle with tick molecules formulated as antigens to elicit a protective immune response is a strategy proven useful for the integrated control of cattle ticks [7,10,36]. The benefits of using anti-tick vaccines as part of an integrated control program include a reduction in the use of acaricides, extending the useful life of acaricides by delaying the onset of resistance, reducing the incidence of *R. microplus*-borne diseases, and decreased production costs [6,8,9]. The only tick molecule currently developed and marketed as a component of an anti-tick vaccine is Bm86 from *R. microplus*. Bm86 is a glycoprotein expressed in eggs a few days after oviposition, unfed and blood-fed larvae, nymphs, adult males, and in the ovaries of partially engorged adult females [11]. The Bm86 gene appeared to be down-regulated in the ovaries of ticks feeding on cattle infected with *B. bovis* [12]. Anti-tick vaccine products based on the recombinant version of Bm86 (rBm86) were registered in Australia under the trade name TickGARD®, and in Cuba as Gavac® in the 1990s [13,36]. The rBm86-based vaccines are highly efficacious against *R. annulatus* [38,39], but the level of efficacy against *R. microplus* varies according to characteristics of the tick population targeted and host factors among other things [14,15]. Pen trials conducted in the state of Mato Grosso do Sul, Brazil revealed that the efficacy of Bm86-based vaccines against the Campo Grande strain of *R. microplus* ranged from 31 to 49% [17,18].

* Corresponding author. Tel.: +1 830 792 0304; fax: +1 830 792 0302.
E-mail addresses: andreott@cnpgc.embrapa.br (R. Andreotti), Rodrigocunha@cnpgc.embrapa.br (R.C. Cunha), msoares_mari@hotmail.com (M.A. Soares), felix.guerrero@ars.usda.gov (F.D. Guerrero), Fabio@leivasleite.com.br (F.P. Leivas Leite), beto.perezdeleon@ars.usda.gov (A.A. Pérez de León).

0264-410X Published by Elsevier Ltd. Open access under the Elsevier OA license.
http://dx.doi.org/10.1016/j.vaccine.2012.08.066

Efficacy around 99% against *R. annulatus* obtained with Bm86-based vaccines is an indication of the consistent high level of anti-*R. microplus* immunoprotection that a novel antigenic and immunogenic tick molecule, or combinations thereof, could elicit in vaccinated cattle. Such level of efficacy offers the opportunity to incorporate vaccination as a tool for the integrated eradication of cattle fever tick populations [40,41]. The search for protective antigens that are highly efficacious against *R. microplus* continues. Proteinase inhibitors have received attention as a group of molecules found in ticks with potential for use as immunogens in an anti-tick vaccine. Several trypsin inhibitors that are present in the egg, larval and adult stages of *R. microplus* have been described [19–21]. It has been suggested that the *R. microplus* serine protease inhibitors may be involved in larval attachment at the bite site and blood feeding [22]. Trypsin inhibitors from *R. microplus* larvae purified in their native form elicited a protective immune response in vaccinated cattle yielding 72.8% efficacy, and 69.7% reduction in the number of adult female ticks completing the parasitic phase of their life cycle [22]. However, a peptide designed from one of the *R. microplus* larval trypsin inhibitors afforded only 18.4% immunoprotection against tick infestation in crossbred cattle [23].

The use of recombinant trypsin inhibitors can circumvent the challenge of having to purify trypsin inhibitors in sufficient quantities to conduct cattle tick vaccination tests [21,22]. An expressed sequence tag originally identified in *R. microplus* larvae was later reported to correspond to sequence amplified from ovarian tissue coding for the fragment of a Kunitz-BPTI domain protease inhibitor termed rBmTI-6 [21,24]. The rBmTI-6 was expressed in the *Pichia pastoris* system and characterized as a three-headed Kunitz-bovine pancreatic trypsin inhibitor, but its ability to protect immunized cattle against tick infestation remained to be determined [21]. Here, the partial nucleotide sequence of the putative *R. microplus* larval trypsin inhibitor was used to produce the recombinant polypeptide in the yeast expression system to probe its immunoprotective properties [24]. Results of the cattle immunization trial and other experiments using the recombinant *R. microplus* larval trypsin inhibitor (rRmLTI) are also reported.

2. Materials and methods

2.1. Ticks

Ticks used for this study were obtained from a laboratory colony maintained at EMBRAPA Beef Cattle. This colony was established using ticks collected from bovines in ranches around Campo Grande, Matto Grosso do Sul, Brazil. Ticks were maintained under laboratory conditions for two years prior to use in the experiments reported here. Cattle were used to cycle the tick progeny. Tick stages requiring incubation were kept in the laboratory at 28 °C and 80% relative humidity. The Campo Grande cattle tick strain is susceptible to commercially available acaricides.

2.2. Transformation of Pichia pastoris with RmLTI DNA construct and sequence analysis

The expressed sequence tag (EST) coding for RmLTI (GenBank ID: CK186726 [21,24]) was optimized for *P. pastoris* codon usage, and synthesized by Epoch Biolabs, Inc. Codon optimization was done using Epoch Biolabs, Inc. proprietary software set at 15% cut off for codon efficiency. This RmLTI DNA fragment was cloned into pPICZαA, producing the pPICZαRmLTI construct. The recombinant plasmid codes for a His tag that is added to the N-terminus of the protein product. Previously described procedures were followed to produce rRmLTI in the *P. pastoris* expression system [25].

Alignment, similarity, and discordance comparisons based on bioinformatics techniques were conducted between predicted amino acid sequences for: rRmLTI, EST CK186726, BmTI-6 from ovarian cDNA (GenBank ID: P83606.2), and N-terminal amino acid sequence information for BmTI-A (GenBank ID: P83609), BmTI-D (GenBank ID: P83607), BmTI-2 (GenBank ID: P83603), and BmTI-3 (GenBank ID: P83604). ClustalW from the BioEdit suite was used with Vector NTI® software (Invitrogen) as described previously to conduct the bioinformatics analyses [16]. The amino acid sequence from rRmLTI was submitted to protein function and superfamily analysis using the protein domains identifier software InterProScan [42].

2.3. rRmLTI quantitation and purification, and murine antibody production

Protein concentration in *P. pastoris* culture supernatant was quantified as described previously [25]. Proteins were precipitated with methanol and the precipitated proteins resuspended in denaturing binding buffer (8 M Urea, 20 mM sodium phosphate pH 7.8, 500 mM NaCl). The rRmLTI was purified using a Ni^{2+} charged Ni-NTA (Qiagen, Hilden, Germany) affinity column with denaturing elution buffer (8 M Urea, 20 mM Sodium Phosphate pH 4.0, 500 mM NaCl) and the purification process monitored by 7.5% SDS-PAGE. Eluted fractions of high purity were pooled and dialyzed against PBS.

Animal care and use was conducted at EMBRAPA Beef Cattle according to institutional guidelines. Polyclonal serum against *R. microplus* larval extract or rRmLTI was produced using BALB/c mice as described previously [25]. The RmLTI vaccine was prepared with 500 μg of rRmLTI protein resuspended in 4 mL of 150-mM Tris–HCl at pH 7.4 and emulsified with 6 mL of Montanide ISA 61 VG (Seppic, Paris). Twelve female BALB/c mice were used, which were separated into two groups of six animals. One group received the rRmLTI formulation and the other the larval extract preparation. Each mouse within the respective group was immunized with 50 $\mu g\,mL^{-1}$ $dose^{-1}$ of rRmLTI, or 100 $\mu g\,mL^{-1}$ $dose^{-1}$ of larval extract. Three subcutaneous doses were applied at 21-day intervals. Sera were collected 21 days after the last dose.

2.4. rRmLTI electrophoresis and Western blotting

Electrophoresis analysis was performed on material from a 10% polyacrylamide gel run with 10 μL of culture supernatant per well containing 0.55 $\mu g\,\mu L^{-1}$ of protein. Subsequent Western blotting analysis was performed as previously described [25].

2.5. Pen trial, bovine serum analysis, and efficacy assessment

The rRmLTI antigen expressed in *P. pastoris* was adjuvated with Montanide ISA 61 VG (Seppic, Paris) and doses of 2 mL containing 100 μg of the recombinant protein prepared. One-year old Holstein calves were randomly distributed into two groups of six animals each. One group was immunized with rRmLTI antigen purified and formulated as described above. The second group (negative controls) was injected with adjuvant/saline alone. Serum samples were collected and processed, and all procedures involving ticks were performed according to methods described previously [17]. Sera obtained immediately before the initial injection, and at different time points thereafter, from each of the six cattle in the vaccinated and control groups were pooled and stored in an ultralow freezer until ready for ELISA testing.

For ELISA, microtiter plates were coated with 1 $\mu g\,mL^{-1}$ of rRmLTI antigen in 20 mM sodium carbonate buffer (pH 9.6), 50 μL per well, and incubated overnight at 4 °C. Duplicate samples of

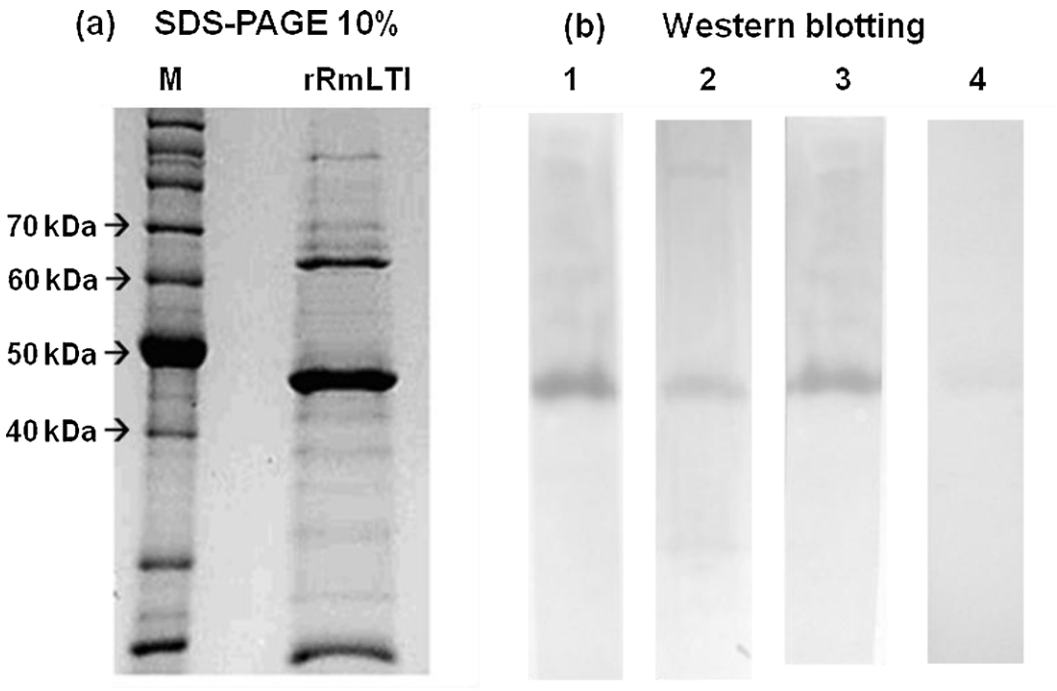


Fig. 1. Identification of *P. pastoris* product as larval trypsin inhibitor present in *R. microplus* larvae by electrophoresis and immunoblotting. Supernatant of culture containing *P. pastoris* transformed with pPICZαA-RmLTI was processed to perform: (a) electrophoresis (SDS-PAGE 10%) where the gel was loaded with M (molecular weight marker [BenchMark™ Protein Ladder, Invitrogen]), and rRmLTI (supernatant of *P. pastoris* culture expressing rRmLTI); (b) Western blot of rRmLTI detected with: (1) anti-His monoclonal antibody, (2) serum from mice vaccinated with rRmLTI, (3) serum from mice vaccinated with larval extract, and (4) serum from unvaccinated mice.

pooled sera for each group and sampling date were tested. Subsequent procedures were performed as described previously [25].

Procedures described before were followed to assess treatment effects on tick biology and vaccine efficacy [17,26]. Engorged female ticks dropping off of cattle from each group were collected daily for 13 days and incubated in the laboratory to obtain eggs that were pooled until 1 gram was accumulated. The egg masses obtained per collection day from several ticks in each group were incubated to determine hatching rates.

2.6. Ex vivo feeding of anti-rRmLTI IgG and assessment of tick egg eclosion rates

Serum samples from bovines immunized with rRmLTI were collected just prior to tick infestation and subjected to affinity chromatography on a protein A-Sepharose column to purify immunoglobulin G (IgG). The IgG eluted from the column resulted in a yield of 5 mg mL^{-1} of non-immune serum. Four groups, each consisting of ten adult female ticks, were set up for treatment. Each tick in the respective group was fed with the antibody mixture containing 0, 25, 50, or 100 μg of purified IgG in 20 μL by placing a capillary tube in its hypostome. The effect on egg eclosion was assessed as described previously [17].

2.7. Statistics

Data on female reproductive parameters were analyzed using a *t*-test. Otherwise, differences between means were determined using one-way analysis of variance (ANOVA). Differences were considered significant when $p < 0.05$.

3. Results

3.1. Production of rRmLTI in P. pastoris expression system

Identity of the DNA insert in pPICZαRmLTI was confirmed by sequencing and alignment with the RmLTI clone sequence. One Mut^+ clone was selected and analysis of the induced recombinant protein revealed a band of approximately 46 kDa. The calculated molecular weight for rRmLTI was 37.9 kDa. It remains to be validated if post-translational modification through glycosylation, potentially adding 8–9 mannose and two N-acetyl-glucosamine molecules, may have caused the discrepancy between the calculated and observed molecular weights of the protein product in *P. pastoris*. Direct quantification from culture supernatants revealed rRmLTI production levels of 550 mg L^{-1}. Analysis of the nickel column purification product showed a protein of 46 kDa and the yield following purification was 870 mg L^{-1}. Western blot analysis of the rRmLTI protein was carried out with primary sera from mice (anti-*R. microplus* larval extract and anti-rRmLTI) and anti-His tag monoclonal antibody revealing affinity for a protein of approximately 46 kDa (Fig. 1).

3.2. Immunoprotection against R. microplus infestation in cattle vaccinated with rRmLTI

The antibody response of cattle immunized with the vaccine formulation containing rRmLTI is shown in Fig. 2. Antibody levels against rRmLTI peaked around 31 days after the second booster immunization. Tick infestations were established around ten days before the apparent decline in the specific antibody response commenced. A transient effect on the average weight of engorged adult female ticks dropping off of vaccinated cattle was apparent through the ninth day of the collection period (Fig. 3). With the exception of days 2 and 4, the average weight of engorged female ticks collected from the vaccinated group was significantly lower up to day nine (Fig. 3; $p < 0.05$). Equivalence of the average engorged adult female tick weight between groups beyond day 9 of the collection period was temporally associated with the aforementioned decline in anti-rRmLTI antibody levels (Fig. 2). A similar tendency was observed in the eclosion rate for eggs collected from ticks detaching from vaccinated cattle (Fig. 4).

Table 1
Efficacy of vaccine containing recombinant *R. microplus* larval trypsin inhibitor against ticks infesting cattle and its effect on female reproductive parameters.

Animal	Tick total number		Tick mean weight (mg)		Egg mean weight (mg)		Larval hatchability (%)	
	Control	Vaccinated	Control	Vaccinated	Control	Vaccinated	Control	Vaccinated
1	1209	818	267	210	112	161	95	90
2	661	441	254	209	138	142	98	85
3	571	588	276	209	121	143	97	86
4	521	602	285	234	145	160	98	89
5	1089	671	304	209	160	154	96	91
6	543	89	275	190	149	125	96	87
Mean ± SD[a]	766 ± 303	535 ± 250	277 ± 17	210 ± 14	138 ± 18	148 ± 014	97 ± 1.2	88 ± 2.4
t-Test	$p = 0.1810$		$p < 0.0001$		$p = 0.3040$		$p < 0.0001$	
% Reduction[b]	DT = 30.15		DW = 24.66		DO = −7.27		DF = 8.97	
Efficacy = 100 [1 − (535/766 × 148/138 × 88/97)] = 32%								

[a] Arithmetic mean ± standard deviation; *p*-values of *t*-test for independent samples are shown.
[b] Percent reduction was calculated in relation to the control unvaccinated group: DT, adult female ticks; DW, tick weight; DO, egg laying capacity; DF, fertility. Efficacy (%) = 100 [1 − (CRT × CR0 × CRF)]; where CRT: reduction in the number of adult female ticks, CRO: reduction in the egg laying capacity, CRF: reduction in fertility.

The cumulative count of engorged adult female ticks collected up to day 13 after detachment started was used to calculate the effects of vaccination with rRmLTI (Table 1). Vaccinated cattle had 30% less ticks detaching from them than the animals injected with adjuvant only. Although egg laying capacity was unaffected, there was a significant effect associated with vaccination on tick weight and larval hatchability (Table 1; $p < 0.05$). Overall, the rRmLTI vaccine afforded 32% immunoprotection against cattle tick infestation (Table 1).

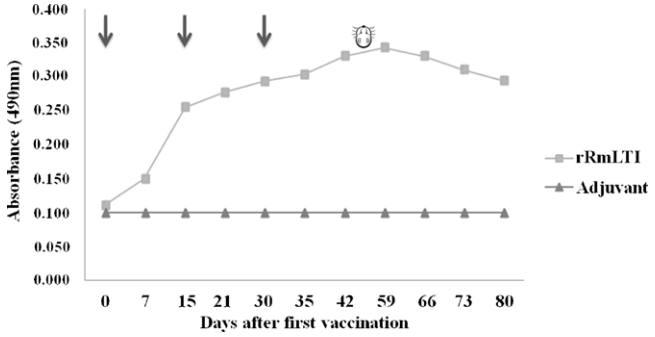


Fig. 2. Profile of humoral immune response in cattle vaccinated with rRmLTI. Absorbance readings are for ELISA tests of weekly antisera samples collected from two groups of six cattle immunized at 0, 15, and 30 days (arrows) with rRmLTI (squared line) or adjuvant (triangled line). Tick icon indicates day 51 after the first vaccination, which is when cattle were infested. Antibody titers of immunized cattle are depicted as the $OD_{490\,nm}$ value of the 1:100 dilution of serum samples. Individual samples in each group collected on the day shown in the figure were pooled for ELISA testing.

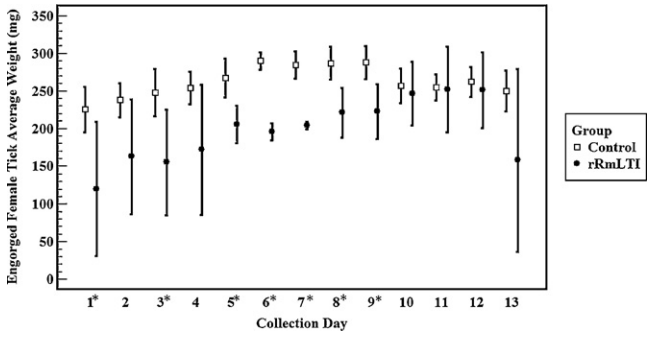


Fig. 3. Reduced weight of female *R. microplus* ticks feeding on cattle vaccinated with rRmLTI. *Group means ± SD are shown. Collection day with asterisk indicates the average weight between engorged female ticks feeding on vaccinated or control cattle was significantly different ($p < 0.05$).

3.3. Effect of purified anti-rRmLTI IgG on tick egg eclosion

The effect of the anti-rRmLTI antibody response on egg hatching was explored further *ex vivo*. An inverse dose-response was observed between egg hatching and the amount of IgG imbibed by the gravid tick (Fig. 5). The viability of eggs laid by female ticks ingesting IgG antibodies from cattle vaccinated with rRmLTI was significantly compromised and hatching decreased 75.6% in eggs from ticks fed 100 μg of IgG ($p < 0.05$).

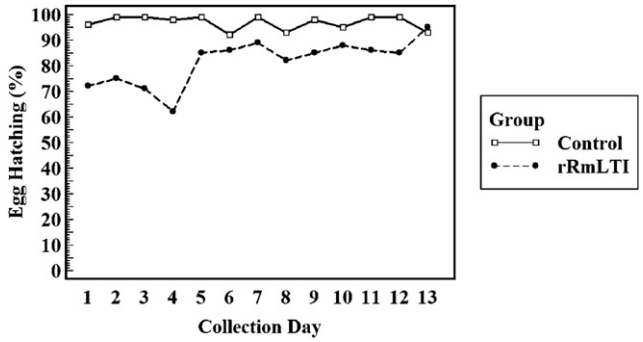


Fig. 4. Percent hatch of eggs laid by female *R. microplus* ticks feeding on control cattle or those vaccinated with rRmLTI. Refer to text for details on egg processing.

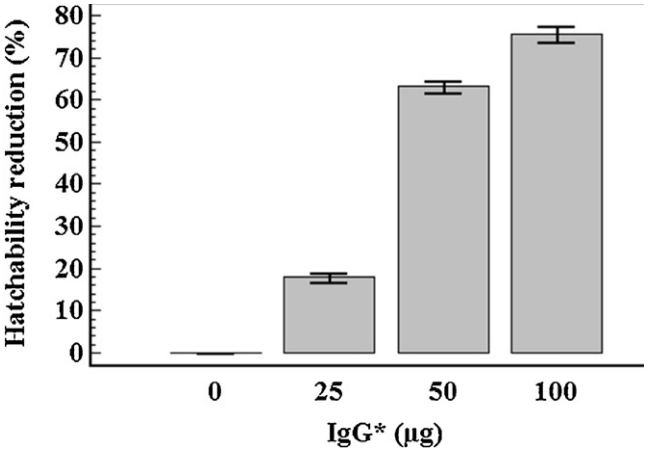


Fig. 5. Anti-rRmLTI IgG imbibed by female cattle ticks inhibits egg hatching in a dose-dependent manner. *Four groups of ten ticks received 0, 25, 50 and 100 μg of purified IgG. Each tick was fed the specified amount of IgG in a volume of 20 μL using a capillary tube. Treatment means ± SD are shown. Mean percent hatch inhibition was significantly different between the treatment groups.

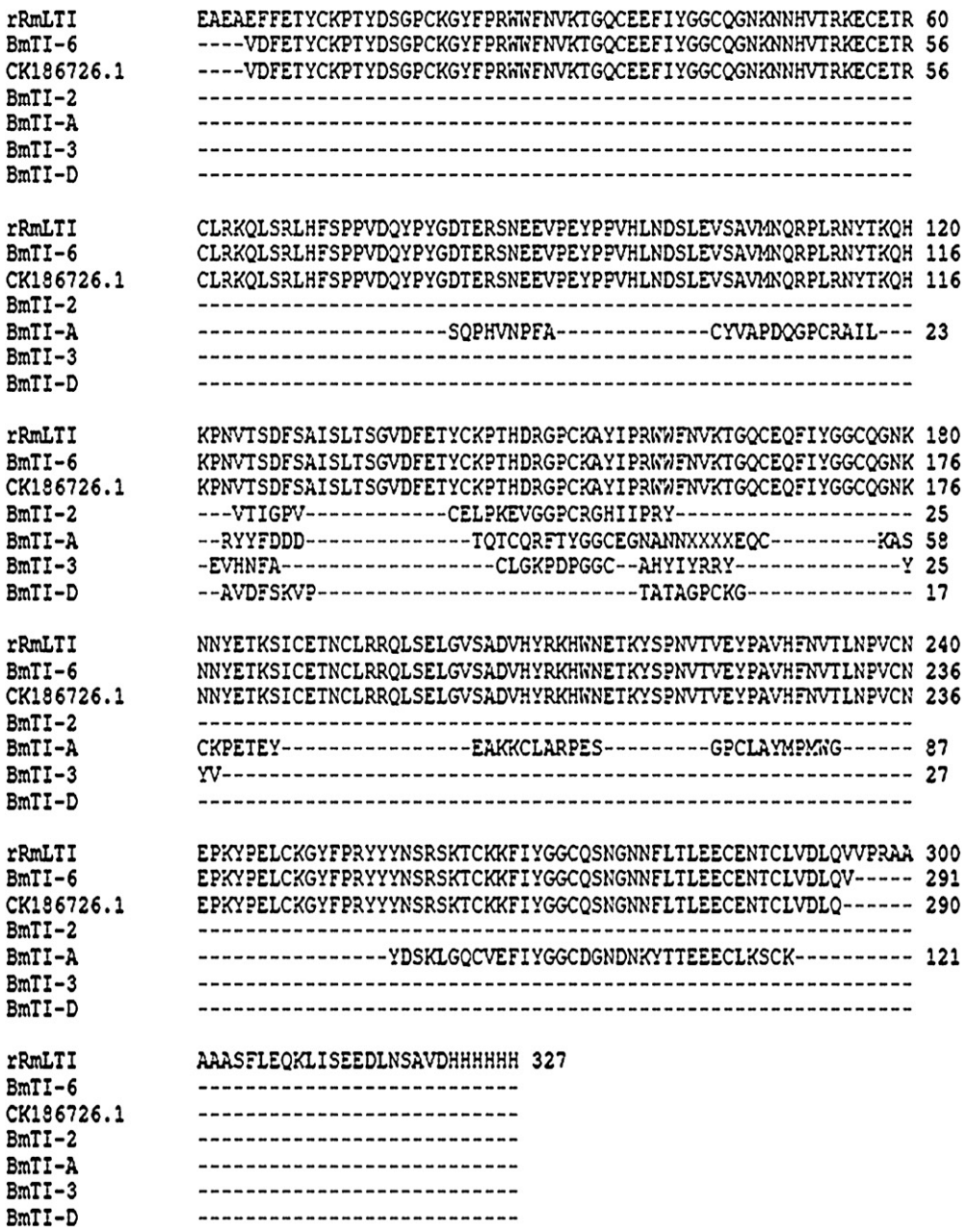


Fig. 6. Alignment of predicted amino acid sequences for rRmLTI, EST CK186726, BmTI-6 from ovarian cDNA, and N-terminal amino acid sequence information for BmTI-A, BmTI-D, BmTI-2, and BmTI-3.

3.4. Identification of RmLTI as a member of the Kunitz-bovine pancreatic trypsin inhibitor family

A comparison of the DNA sequences from the EST CK186726 and the RmLTI clone optimized for codon usage in *P. pastoris* revealed 77% identity between the two sequences. The RmLTI DNA sequence in the yeast expression system was missing nineteen bases of the corresponding EST sequence (data not shown).

Fig. 6 depicts the alignment of predicted amino acid sequences for rRmLTI, EST CK186726, BmTI-6 from ovarian cDNA, and N-terminal amino acid sequence information for BmTI-A, BmTI-D, BmTI-2, and BmTI-3. The deduced amino acid sequences between rRmLTI, EST CK186726, and BmTI-6 are 99% identical. Nucleic acid sequence coding for six additional amino acids (EAEAEF) in the N-terminus, and thirty two amino acids (VPRAAAAASFLEQKLISEEDLNSAVDHHHHHH) in the C-terminal portion of the putative rRmLTI product was added during cloning procedures to allow insertion of a restriction site and coding sequence for the poly-His peptide.

The similarity between their partial amino acid sequences suggested that RmLTI in larvae is a member of the Kunitz-bovine pancreatic trypsin inhibitor (BPTI) family like BmTI-6 in the ovary of adult female cattle ticks. Further exploration of the putative function of RmLTI is reflected in Fig. 7. Relevant protein signature features identified in the deduced amino acid sequence encoded in the expressed sequence tag CK186726 include three putative Kunitz-BPTI domains and two putative Kunitz proteinase inhibitor I2 conserved sites. As noted in BmTI-6, six N-glycosylation sites were present in the partial protein sequence of RmLTI. Six cysteine residues were observed within each of the three Kunitz domains, which are thought to form disulfide linkages contributing stability to the compact polypeptide in its folded form.

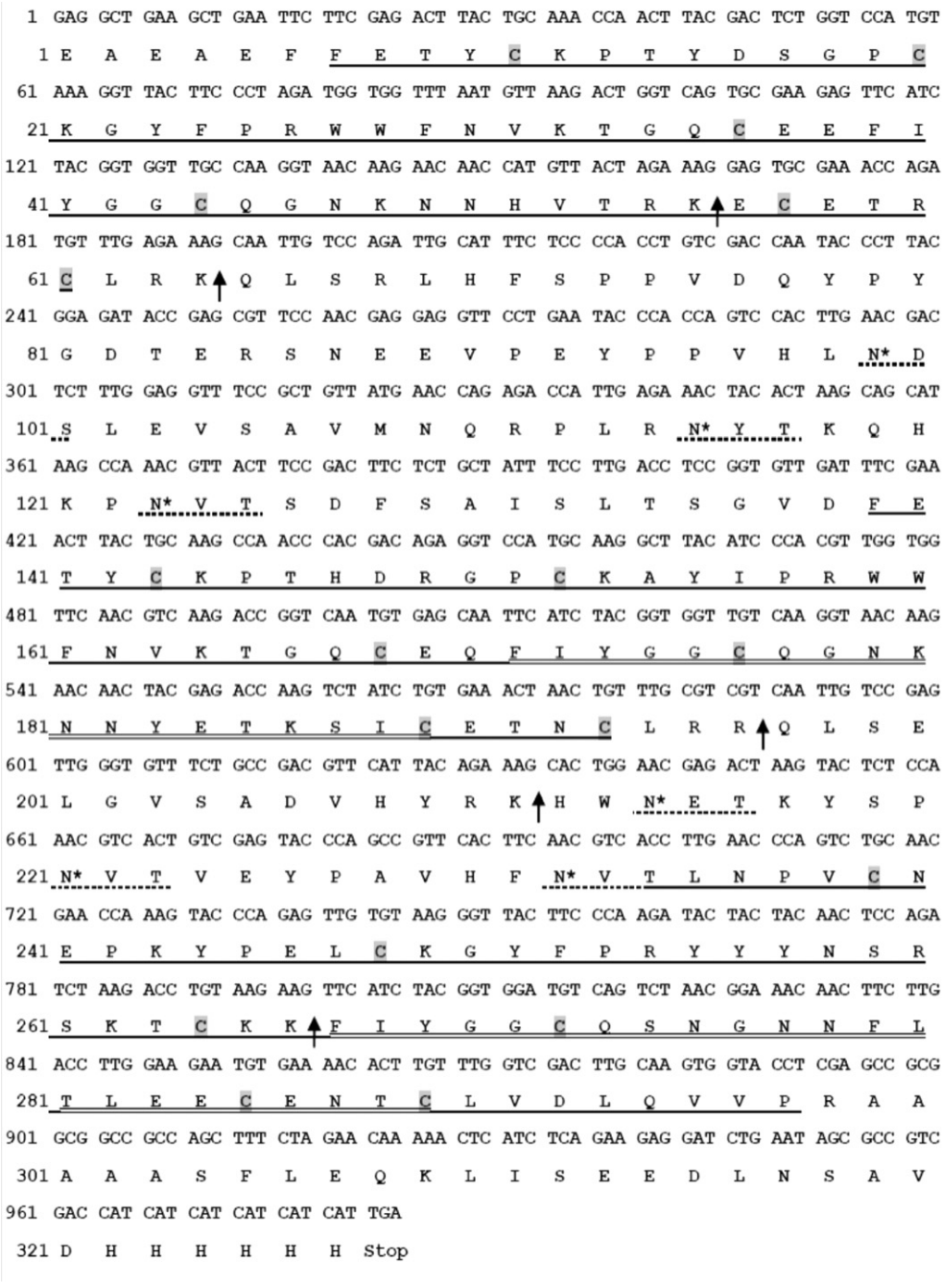


Fig. 7. A trypsin inhibitor in *R. microplus* larvae is encoded in expressed sequence tag CK186726. The amino acid sequence deduced from the RmLTI DNA clone optimized for use in *P. pastoris* was scanned for protein signature features using the InterProScan informatics tool. Asparagine residues (N) predicted to be N-glycosylated are marked with asterisks. A dotted line underlines Asn-Xaa-Ser/Thr codons. Cysteine residues (C) predicted to form disulfide bonds are highlighted in gray. Amino acids encompassing putative Kunitz-BPTI domains are underlined. Two predicted Kunitz proteinase inhibitor I2 conserved sites are double-underlined.

4. Discussion

Assessment of the efficacy against cattle tick infestation in bovines using a vaccine containing the recombinant form of a member of the Kunitz-BPTI family from *R. microplus* produced in the *P. pastoris* expression system is reported for the first time here. A specific and robust humoral immune response against rRmLTI was achieved with the vaccination protocol consisting of three immunizations, each applied every two weeks. The 32% efficacy obtained with the rRmLTI formulation reflects the significant challenge of discovering highly efficacious antigens protecting cattle against *R. microplus* infestation. Vaccination experiments where *Bos indicus* cattle were immunized with a mixture of purified larval trypsin inhibitors containing one or two Kunitz-type domains afforded 72.8% efficacy against *R. microplus* infestation [22]. In contrast, the level of immunoprotection obtained in crossbred cattle vaccinated with the synthetic polypeptide containing 29 aa residues derived from the N terminus of the *R. microplus* trypsin inhibitor A was 18.4% [23]. As the gene encoding RmLTI remains to be fully characterized, the apparent discrepancy between specific antibody levels and the low level of efficacy obtained with the rRmLTI vaccine may be due to the partial gene sequence of the EST used to produce

the recombinant protein product in the yeast expression system. Alternatively, the structural and functional redundancy in proteins belonging to the Kunitz family present in *R. microplus* may also account for the relatively low efficacy attained with the rRmLTI vaccine.

Although binding of rRmLTI by polyclonal antibodies from mice immunized with tick larva extract indicates that the recombinant polypeptide produced in *P. pastoris* was as antigenic as the native form of the cognate larval trypsin inhibitor, it is possible that those antibodies recognized epitopes shared by the several trypsin inhibitors discovered in *R. microplus* larvae. Antiserum from cattle vaccinated with purified *R. microplus* trypsin inhibitors recognized rBmTI-6 produced in *P. pastoris* [21]. Antigenic similarity apparently extends beyond intra-specific boundaries because antiserum against the native form of *R. microplus* larval trypsin inhibitors cross-reacts with trypsin inhibitors identified in *R. sanguineus* larvae [27]. Immunogenicity of the rRmLTI is reflected in the kinetics of the bovine humoral immune response. The significant effect on the rate of larvae hatching from eggs laid by female ticks parasitizing vaccinated cattle, which was amplified by feeding female ticks with purified anti-rRmLTI IgG suggests that potentiation of the humoral response, perhaps using other adjuvants, could enhance the efficacy of a polyvalent vaccine with Kunitz inhibitors from *R. microplus*. Adjuvant choice was shown to influence antibody levels, which correlated with the level of inhibition on malaria parasites [28]. However, no direct correlation was observed between antibodies against rRmLTI and overall efficacy in our study. By comparison, the vast array of Kunitz type inhibitors present in *R. microplus* was invoked to explain the apparently small impact silencing the gene coding for boophilin, a double Kunitz type thrombin inhibitor expressed in the gut, had on egg production [29].

Considering the purported involvement of larval trypsin inhibitors and confirmed role of other Kunitz inhibitors in blood feeding, the reduced number of female ticks detaching from vaccinated cattle may reflect the impact of bovine anti-rRmLTI antibodies on the ability of *R. microplus* to acquire a blood meal [20,29]. However, the physiological roles of RmLTI and BmTI-6 remain to be determined in the larval and adult stages of the cattle tick, respectively, despite similarities in their partial nucleotide and amino acid sequences. Without knowing the function of RmLTI and BmTI-6, it remains possible that the decrease in hatching rates observed in eggs laid by female ticks fed purified IgG antibodies obtained from vaccinated cattle resulted from the effects of antibody binding to epitopes shared by rRmLTI and the native form of BmTI-6 in *R. microplus* ovaries.

The Kunitz family of polypeptides is one of at least 20 families belonging to the canonical type of serine protease inhibitors [30]. A characteristic of proteins belonging to this family is the Kunitz domain that can be present in single or multiple copies. At least 303 Kunitz proteins have been identified in ticks thus far and some of them can contain as many as seven Kunitz domains [31]. Cysteine patterns were used to classify Kunitz proteins into three groups to facilitate study of their evolution in the Lyme disease vector *Ixodes scapularis* and to expand the analysis to ticks in general [31]. Although primarily involved in proteinase inhibition, the Kunitz domain has evolved to perform other functions requiring protein-protein interactions [32]. Cattle tick ovaries, fat body, hemocytes, and midgut contain Kunitz proteins [21,29,33,34]. Proteomic studies revealed the presence of Kunitz proteins that are up-regulated in ovarian tissue when *R. microplus* is infected with *Babesia bovis* [35]. A publicly available genomic database called CattleTickBase offers the opportunity to study the evolutionary history of Kunitz proteins in *R. microplus* [35]. It is possible that BmTI-6 and the RmLTI encoded by CK186726 are splice variants of the same gene or paralogs of the same Kunitz protein as suggested before for BmTI-A and other Kunitz proteins present in cattle tick ovary [34].

Previous research documenting 72.8% efficacy against *R. microplus* infestation using purified trypsin inhibitors and the critical role Kunitz proteins play in various biological processes including proteinase inhibition warrant continued vaccine discovery research with this protein family. Production of rRmLTI in *P. pastoris* facilitates its use to formulate polyvalent cattle tick vaccines that include other Kunitz proteins or different antigens from *R. microplus*. The level of immunoprotection attained through vaccination with rRmLTI was low as compared to other novel antigens discovered recently [37,43]. Of note are the results from vaccination using immunogenic peptides that yielded tick efficacy between 80 and 90% [44,45]. Salivary glands, midgut, and ovaries are prime targets to disrupt cattle tick biology using vaccines and Kunitz proteins are abundant in those tissues. The use of epitopes from Kunitz proteins in combination with immunogenic portions of other tick molecules to produce a dual action vaccine could be another way to exploit the redundancy of *R. microplus* Kunitz inhibitors to innovate a highly efficacious cattle tick vaccine.

Acknowledgments

Embrapa Beef Cattle, CNPq, and Fundect are gratefully acknowledged for financial support. This article reports the results of research only. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation of endorsement by the U.S. Department of Agriculture. F.D. Guerrero and A.A. Pérez de León are funded by USDA-ARS appropriated project 6205-32000-031-00D. The U.S. Department of Agriculture is an equal opportunity provider and employer.*Conflict of statement:* The authors declare that they have no competing interests. *Authors contributions*: RA developed proposal that was funded to test the immunoprotection of trypsin inhibitors from cattle tick larvae and helped prepare the article. RCC conducted research, and acquired and analyzed data related to DNA cloning and production of rRmLTI in the yeast expression system. MAS maintained the cattle tick colony, conducted and acquired data from the stall test, and supported laboratory experiments involving the purification of rRmLTI. FDG assisted with the bioinformatics analysis and interpretation of data related to the BmTI EST sequence, and article preparation. FPLL contributed to study design for polyclonal antibody production, murine serum sample collection, and immune response analysis. AAPL co-developed proposal funded to test the immunoprotection of trypsin inhibitors from cattle tick larvae, analyzed and interpreted the data, and drafted the article. All authors approved the final version of the manuscript submitted for publication.

References

[1] Grisi L, Massard CL, Moya-Borja GE, Pereira JB. Impacto das principais ectoparasitoses em bovinos no Brasil. A Hora Veterinária 2002;125:8–10.
[2] De La Fuente J, Kocan KM, Almazan NC, Blouin EF. Targeting the tick–pathogen interface for novel control strategies. Front Biosci 2008;13:6947–56.
[3] Guerrero FD, Lovis L, Martins JR. Acaricide resistance mechanisms in *Rhipicephalus* (*Boophilus*) *microplus*. Rev Bras Parasitol Vet 2012;21:1–6.
[4] Andreotti R, Guerrero FD, Soares MA, Barros JC, Miller RJ, Pérez de León A. Acaricide resistance of *Rhipicephalus* (*Boophilus*) *microplus* in State of Mato Grosso do Sul, Brazil. Rev Bras Parasitol Vet 2011;20:127–33.
[5] Rosario-Cruz R, Almazan C, Miller RJ, Dominguez-Garcia DI, Hernandez-Ortiz R, De La Fuente J. Genetic basis and impact of tick acaricide resistance. Front Biosci 2009;14:2657–65.
[6] De La Fuente J, Kocan KM, Blouin EF. Tick vaccines and the transmission of tick-borne pathogens. Vet Res Commun 2007;1:85–90.
[7] Willadsen P, Kemp DH. Vaccination with 'concealed' antigens for tick control. Parasitol Today 1988;4:196–8.
[8] Rodríguez M, Penichet ML, Mouris AE, Labarta V, Lorenzo Luaces L, Rubiera R, et al. Control of *Boophilus microplus* populations in grazing cattle vaccinated with a recombinant Bm86 antigen preparation. Vet Parasitol 1995;57:339–49.

[9] De La Fuente J, Rodriguez M, Redondo M, Montero C, Garcia-Garcia JC, Mendez L, et al. Field studies and cost-effectiveness analysis of vaccination with Gavac against the cattle tick *Boophilus microplus*. Vaccine 1998;16:366–73.
[10] Redondo M, Fragoso H, Ortíz M, Montero C, Lona J, Medellín JA, et al. Integrated control of acaricide-resistant *Boophilus microplus* populations on grazing cattle in Mexico using vaccination with Gavac and amidine treatments. Exp Appl Acarol 1999;23:841–9.
[11] Nijhof AM, Balk JA, Postigo M, Jongejan F. Selection of reference genes for quantitative RT-PCR studies in *Rhipicephalus* (*Boophilus*) *microplus* and *Rhipicephalus appendiculatus* ticks and determination of the expression profile of Bm86. BMC Mol Biol 2009;10:112.
[12] Bastos RG, Ueti MW, Knowles DP, Scoles GA. The *Rhipicephalus* (*Boophilus*) *microplus* Bm86 gene plays a critical role in the fitness of ticks fed on cattle during acute *Babesia bovis* infection. Parasites Vectors 2010;3:1–11.
[13] Willadsen P. Antigen cocktails: valid hypothesis or unsubstantiated hope. Trends Parasitol 2008;4:164–7.
[14] García-García JC, Montero C, Redondo M, Vargas M, Canales M, Boue O, et al. Control of ticks resistant to immunization with Bm86 in cattle vaccinated with the recombinant antigen Bm95 isolated from the cattle tick, *Boophilus microplus*. Vaccine 2000;18:2275–87.
[15] Sossai S, Peconick AP, Sales-Junior PA, Marcelino FC, Vargas MI, Neves ES, et al. Polymorphism of the bm86 gene in South American strains of the cattle tick *Boophilus microplus*. Exp Appl Acarol 2005;37(3-4):199–214.
[16] Andreotti R, Pedroso MS, Caetano AR, Martins NF. Comparison of predicted binders in *Rhipicephalus* (*Boophilus*) *microplus* intestine protein variants Bm86 Campo Grande strain, Bm86 and Bm95. Rev Bras Parasitol Vet 2008;17:93–8.
[17] Andreotti R. Performance of two Bm86 antigen vaccin formulation against tick using crossbreed bovines in stall test. Rev Bras Parasitol Vet 2006;15:97–100.
[18] Cunha RC, Pérez de León AA, Leite FPL, da Silva Pinto L, dos Santos Júnior AG, Andreotti R. Bovine immunoprotection against *Rhipicephalus microplus* with recombinant Bm86-Campo Grande antigen. Rev Bras Parasitol Vet 2012;21:1–9.
[19] Tanaka AS, Andreotti R, Gomes A, Torquato RJS, Sampaio MU, Sampaio CAM. A double headed serine proteinase inhibitor – human plasma kallikrein and elastase inhibitor – from *Boophilus microplus* larvae. Immunopharmacology 1999;45:171–7.
[20] Andreotti R, Malavazi-Piza KC, Sasaki SD, Torquato RJ, Gomes A, Tanaka AS. Serine proteinase inhibitors from eggs and larvae of tick *Boophilus microplus*: purification and biochemical characterization. J Protein Chem 2001;20:337–43.
[21] Sasaki SD, Tanaka AS. rBmTI-6, a Kunitz-BPTI domain protease inhibitor from the tick *Boophilus microplus*, its cloning, expression and biochemical characterization. Vet Parasitol 2008;155:133–41.
[22] Andreotti R, Gomes A, Malavazi-Piza KC, Sasaki SD, Sampaio CA, Tanaka AS. BmTI antigens induce a bovine protective immune response against *Boophilus microplus* tick. Int Immunopharmacol 2002;2:557–63.
[23] Andreotti R. Synthetic bmti n-terminal fragment as antigen in bovine immunoprotection against the tick *Boophilus microplus* in a pen trial. Exp Parasitol 2007;116:66–70.
[24] Guerrero FD, Miller RJ, Rousseau ME, Sunkara S, Quackenbush J, Lee Y, et al. BmiGI: a database of cDNAs expressed in *Boophilus microplus*, the tropical/southern cattle tick. Insect Biochem Mol Biol 2005;35:585–95.
[25] Cunha RC, Andreotti R, Leite FPL. *Rhipicephalus* (*Boophilus*) *microplus*: expression and characterization of Bm86-CG in *Pichia pastoris*. Rev Bras Parasitol Vet 2011;20:103–10.
[26] Garcia-Garcia JC, Gonzalez IL, Gonzalez DM, Valdes M, Mendez L, Lamberti J, et al. Sequence variations in the *Boophilus microplus* Bm86 locus and implications for immunoprotection in cattle vaccinated with this antigen. Exp Applied Acarol 1999;23:883–95.
[27] Azzolini SSA, Sasaki SD, Torquato RJ, Andreotti R, Andreotti E, Tanaka AS. Rhipicephalus sanguineus trypsin inhibitors present in the tick larvae: isolation, characterization, and partial primary structure determination. Arch Biochem Biophys 2003;417:176–82.
[28] Kusi KA, Faber BW, Riasat V, Thomas AW, Kocken CH, Remarque EJ. Generation of humoral immune responses to multi-allele PfAMA1 vaccines; effect of adjuvant and number of component alleles on the breadth of response. PLoS ONE 2010;5:e15391, http://dx.doi.org/10.1371/journal.pone.0015391.
[29] Soares TS, Watanabe RM, Tanaka-Azevedo AM, Torquato RJ, Lu S, Figueiredo AC, et al. Expression and functional characterization of boophilin, a thrombin inhibitor from *Rhipicephalus* (*Boophilus*) *microplus* midgut. Vet Parasitol 2012;January [Epub ahead of print].
[30] Krowarsch D, Cierpicki T, Jelen F, Otlewski J. Canonical protein inhibitors of serine proteases. Cell Mol Life Sci 2003;60:2427–44.
[31] Dai SX, Zhang AD, Huang JF. Evolution, expansion and expression of the Kunitz/BPTI gene family associated with long-term blood feeding in *Ixodes scapularis*. BMC Evol Biol 2012;12:4.
[32] MacLean 2nd JA, Roberts RM, Green JA. Atypical Kunitz-type serine proteinase inhibitors produced by the ruminant placenta. Biol Reprod 2004;71:455–63.
[33] Lima CA, Torquato RJ, Sasaki SD, Justo GZ, Tanaka AS. Biochemical characterization of a Kunitz type inhibitor similar to dendrotoxins produced by *Rhipicephalus* (*Boophilus*) *microplus* (Acari: Ixodidae) hemocytes. Vet Parasitol 2010;167:279–87.
[34] Rachinsky A, Guerrero FD, Scoles GA. Differential protein expression in ovaries of uninfected and Babesia-infected southern cattle ticks, *Rhipicephalus* (*Boophilus*) *microplus*. Insect Biochem Mol Biol 2007;37:1291–308.
[35] Bellgard MI, Moolhuijzen PM, Guerrero FD, Schibeci D, Rodriguez-Valle M, Peterson DG, et al. CattleTickBase: an integrated Internet-based bioinformatics resource for *Rhipicephalus* (*Boophilus*) *microplus*. Int J Parasitol 2012;42:161–9.
[36] de la Fuente J, Almazán C, Canales M, Pérez de la Lastra J, Kocan K, Willadsen P. A ten-year review of commercial vaccine performance for control of tick infestations on cattle. Anim Health Res Rev 2007;8:23–8.
[37] Guerrero FD, Miller R, Pérez de León AA. Anti-cattle tick vaccines: many candidate antigens, but will a commercially viable product emerge? Int J Parasitol 2012;42:421–7.
[38] Fragoso H, Rad PH, Ortiz M, Rodríguez M, Redondo M, Herrera L, et al. Protection against *Boophilus annulatus* infestations in cattle vaccinated with the *B. microplus* Bm86-containing vaccine Gavac. Vaccine 1998;16:1990–2.
[39] Pipano E, Alekceev E, Galker F, Fish L, Samish M, Shkap V. Immunity against *Boophilus annulatus* induced by the Bm86 (Tick-GARD) vaccine. Exp Appl Acarol 2003;29:141–9.
[40] Miller R, Estrada-Peña A, Almazán C, Allen A, Jory L, Yeater K, et al. Exploring the use of an anti-tick vaccine as a tool for the integrated eradication of the cattle fever tick *Rhipicephalus* (*Boophilus*) *annulatus*. Vaccine 2012;June [Epub ahead of print].
[41] Pérez de León AA, Teel PD, Auclair AN, Messenger MT, Guerrero FD, Schuster G, et al. Integrated strategy for sustainable cattle fever tick eradication in USA is required to mitigate the impact of global change. Front Physiol 2012;3:195–2011.
[42] Quevillon E, Silventoinen V, Pillai S, Harte N, Mulder N, Apweiler R, et al. InterProScan: protein domains identifier. Nucleic Acids Res 2005;33:116–20.
[43] Parizi LF, Githaka NW, Logullo C, Konnai S, Masuda A, Ohashi K, et al. The quest for a universal vaccine against ticks: cross-immunity insights. Vet J 2012;July [Epub ahead of print].
[44] Rodríguez-Mallon A, Fernández E, Encinosa PE, Bello Y, Méndez-Pérez L, Ruiz LC, et al. A novel tick antigen shows high vaccine efficacy against the dog tick, *Rhipicephalus sanguineus*. Vaccine 2012;30:1782–9.
[45] Patarroyo JH, Vargas MI, González CZ, Guzmán F, Martins-Filho OA, Afonso LC, et al. Immune response of bovines stimulated by synthetic vaccine SBm7462 against *Rhipicephalus* (*Boophilus*) *microplus*. Vet Parasitol 2009;166:333–9.